IDENTIFICATION OF DISSOLVED ORGANIC CHEMICALS IN NATURAL STREAMWATERS OF THE TESUQUE WATERSHED, SANGRE DE CRISTO MOUNTAINS, NEW MEXICO

Rex G. Cates

FINAL REPORT

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INTRODUCTION

Most of the previous interest in the natural organic chemistry of wildland waters has been centered around compounds which cause water to exhibit a yellow to brown color. The literature contains many names such as tannins, phenolics, lignins, humus, ulmin, humic acid, crenic acid, torfic acid, hymatomelic acid, fulvic acid and others, which are used to describe the nature of "colored" waters (Midwood and Felbeck, Jr., 1967; Christman and Ghassemi, 1966; Lamar and Goerlitz, 1966). The organic acids and phenolics are probably the best-known groups in naturally-occurring colored water. Much of the organic matter in these waters consists of a complex mixture of carboxylic acids or their salts that impart a yellow color to the water. Conspicuously missing from the list of compounds in streamwaters are the terpenes (Swift et al., 1979), and attention needs to be given to these compounds.

Many of the qualitative organic studies in the 1960's were limited in scope, and some of the information derived from these studies has been contradictory (Cates and McMurray, 1980).

One reason for the lack of studies on natural dissolved organic acids, bases, and neutrals in wildland streams has been that of unreliable techniques and methodology (Cates and McMurray, 1980). The equipment and techniques used in studies previous to 1974 have sometimes not been desirable even though they were the best available at that time. Other problems exist in changes in the organics during the sample collection, and while the samples were being stored and/or prepared for analysis. In other cases, methods do not remove all of the compounds from the water. Severe

limitations of the method that has become the standard for trace organic analysis are now documented. This method, which is the carbon adsorption method (known in the literature as CAM), only recovers a low percentage of the organic substances from water (Burnham et al., 1973). Additionally, concern has been expressed that sorption and desorption of organic compounds during their sequential extraction is not complete with the CAM method and that the organic compounds may be altered while they are adsorbed on the carbon (Adams et al., 1975). However, with the advent of sufficient techniques, and gas and high pressure liquid chromatography (GLC & HPLC, respectively), organic compounds can be sampled reliably, recovered, and analyzed in their orginal form (Minear et al., 1974; Burnham et al., 1973).

The origin of many of the natural organic molecules is found primarily with the vegetation. Figures 1 and 2 show terpene and phenolic compounds that can be expected to be dissolved in natural waters, especially streams draining watersheds dominated by conifers. Trees and plants in all stages of growth and decay, leaves and needles of ground litter, and the forest soil itself are available for aqueous extraction of organic molecules. The nature and amount of chemicals extracted from vegetation varies from species to species, tissue to tissue (McConnell and Longo, 1971), as well as with the degree of disturbance in the forest (Cates and McMurray, 1980; Christaman and Ghassemi, 1966). The polyphenolic extractives in living woody tissues are partially water soluble at ordinary temperatures and the amount extracted varies greatly with temperature. Aqueous extracts should consist mainly of volatile oils, terpenes, polyhydric alcohols, and a large class of aromatic compounds.

Figure 1. Generalized Terpenoid Pathway. Broken lines suggest several intermediates that are not represented in this figure.

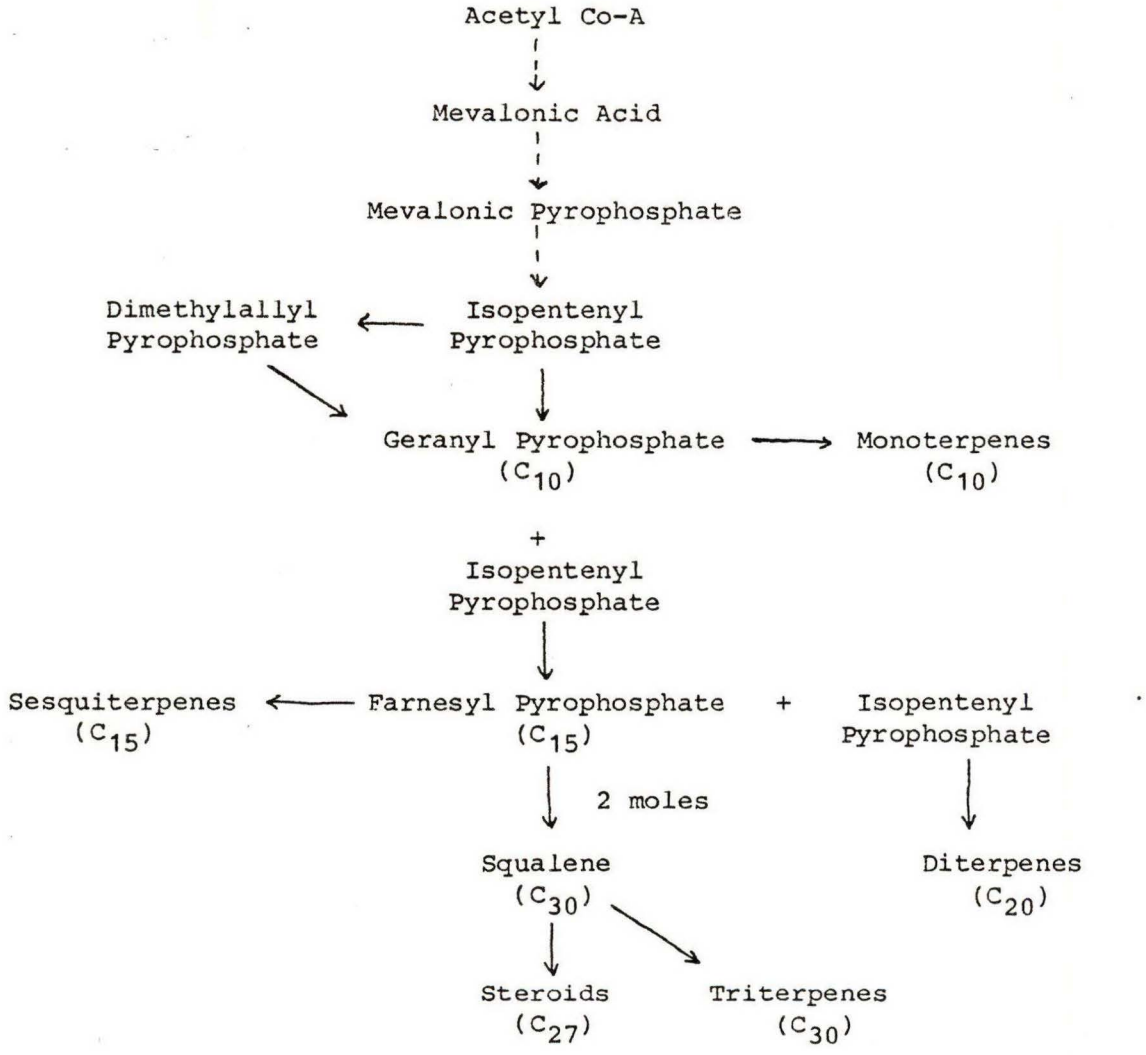
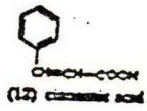


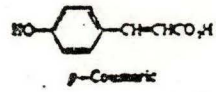
Figure 2. Listing of compounds in major groups of phenolic compounds in Douglas-fir.

Phenylpropanoids

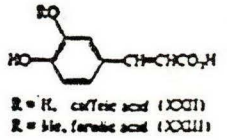
cinnamic acid

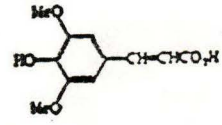


coumaric acid

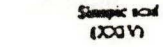


ferulic acid

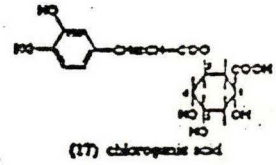




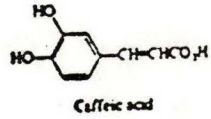
sinapic acid



chlorogenic acid

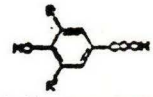


caffeic acid



Benzoic Acids

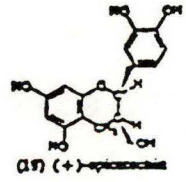
p-hydroxybenzoic

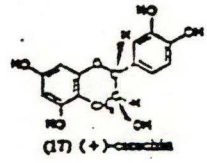


vanillic

Flavonoids

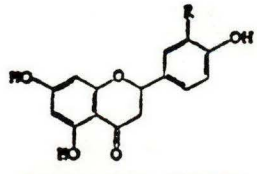
epicatechin ≅ catechin

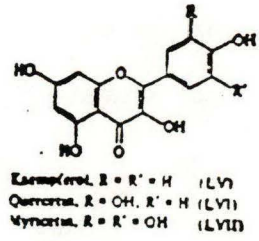




naringenin

dihydrokaempferol





eriodictyol

dihydroquercetin

tannin

polymers

The roles that these compounds play in living and non-living systems are many. Some possess different capabilities to complex with and transport inorganic cations and heavy metals (Shindo and Kuwatsuka, 1977; Brezonik and Klein, 1975; Baker, 1973; Schnitzer and Skinner, 1962). Others are highly toxic to fish and exhibit a variety of effects on mammals and terrestial plants (Horner, 1983; McConnel and Longo, 1971). Phenol itself and a number of its derivatives, in addition to other substances of plant origin such as various elements of croton oil, euphorbia latices, citrus oils, and extracts of tobacco have been shown to act as mammal tumor promoters (Boutwell, 1967). Phenolic acids have been shown to act as plant growth inhibitors (Huang et al., 1976). Additionally, some of these compounds react with chlorine in water-treatment processes forming compounds toxic to both aquatic and terrestial organisms. Rook (1974) and Bellar et al., (1974) discovered that the origin of some of our most toxic substances was due to the interaction between chlorine and these natural organic compounds. The implication of this discovery is not completely known. Both groups found that chlorine, which is used to disinfect natural waters for drinking, reacts with organic substances in the water to form chloroform and other halogenated organics. Chloroform has been reported as a suspect carcinogen (Serle, 1976) and many scientists feel that other trihalomethanes also may be toxic. Trihalomethanes are defined to be trichloromethane (chloroform), bromodichloromethane, dibromochloromethane, and tribromomethane (bromoform) (Glaze, 1977). These appear to be produced by the action of chlorine on humic and fulvic substances (Glaze, 1978).

Cates and McCurray (1980) studied the dissolved organics compounds (acids,

bases, and neutrals) collected from streamwaters draining watersheds in Colorado and New Mexico. The sites chosen were experiencing various levels of disturbances from the development of a new recreational area at Beaver Creek near Vail, Colorado, to small-scale disturbances at the established Santa Fe Ski Basin Recreation area, to non-distrubed sites. The vegetation on the sites consisted of spruce-fir and aspen. At the highly-disturbed Beaver Creek area and during the widening of ski trails at the Santa Fe Ski Basin, significant increases were found in the neutral and acid fractions taken from the streams. Interestingly, once a recreational area was established and not being disturbed, the neutral, acid, and basic fractions were significantly lower than the controls (natural, undisturbed sites of similar elevation and vegetational composition).

In a study carried out in the Tesuque Watershed (Sangre de Cristo Mountains, New mexico), Molles, Gosz, and Cates (1983) report significant increases in the neutral to slightly acidic fraction of aspen streamwater as the result of heavy trampling in experimental plots. Trampling increased this fraction of the dissolved organics in aspen streamwater by 124% as compared to the controls. Aspen lysimeter water, however, was about 40% lower in the neutral to slightly acidic fraction compared to the non-trampled controls. No changes in any dissolved organics were found in spruce-fir streamwater due to trampling. This, however, may have been due to a small sample size as one stream dried up. Trampling was associated with a significant decrease in the neutral content of lysimeters at the spruce-fir site.

There is only a minuscule amount of information on the role and effect

of natural organics of wildland streams in biological phenomena. Furthermore, there is little or no information on the dissolved organic content of lysimeter water, even though significant quantities of these natural organics are present in the water of streams and lysimeters draining disturbed watersheds. This suggests the possibility that other recreational activities involving smaller scale disturbances may affect water quality.

As a result of these studies, and because of a lack of information on the dissolved organic content of natural forest streamwater, a study was undertaken to begin to determine the compounds present in streamwater at the Tesuque Watershed. Specific goals were to:

1. Collect natural water from streams in the Tesuque Watershed;
2. Recover some of the dissolved organic chemicals using various methods;
3 To evaluate these methods with regard to their utility and efficiency;
4. To investigate in some detail the neutral to slightly acidic fraction; and
5. To identify some of the major components of this fraction.

METHODS

Water Collection

Ten 1 of aspen-conifer streamwater were collected on 3 August 1982. This was collected, immediately filtered through 0.45 µm Millipore membrane filters, placed in 3 brown bottles, capped, and transported on ice to the Chemical Ecology Laboratory, Department of Biology, University of New Mexico immediately. The sample was collected from watershed 7 of the Tesuque Watershed Sangre de Cristo Mountains, New Mexico, and was used in the freezing out method.

Another bulk sample of 133 1 of aspen-conifer streamwater was collected at the same watershed on 19 July 1982 in polyethylene carboys. The carboys were packed in ice immediately, transported to the same laboratory, and filtered through 0.45 µm Millipore membrane filters. The filtrate was stored in brown bottles at 4°C in a refrigerator. This was used in the Sep-Pak and sequential election methods.

Freezing Out Method

After filtration 400 ml was placed in a round-bottom flask. The flask, which was connected to a rotary evaporator, was immersed in cooling mixture of ice water and salt at -12°C. After five hours of rotating under a vacuum, the residual liquid was decanted from the solid ice block. Each of the 3 samples was treated in the same manner. Aliquots from each sample were mixed and analyzed as described below. In addition, each of the three samples was analyzed separately on the GLC and HPLC.

Sep-Pak Concentration

This process was undertaken in an effort to maximize recovery of

compounds from the streamwater. The concentration apparatus consisted of mounting 12, 250 ml columns and eight 100 ml burets on ring stands. Each column was fitted with an activated Sep-Pak. The flow rate was adjusted to 250 ml/hr. Each Sep-Pak was loaded with 1000 ml of sample water, and the eluent was refrigerated brown bottles to be used as an acid fraction. Enough tetrabutylamonnium phosphate was added to the Sep-Pak eluent to make 2, 7.5×10^{-2} M solution. This solution was loaded onto activated Sep-Pak cartridges at a rate of 250 ml/hr and a total capacity of 750 ml/cartridge. All loaded cartridges were wrapped in parafilm and refrigerated at 4°C.

Sep-Paks were then eluted with various volumes (flow 0.5 - 4 ml) and concentrations of acetonitrile (from 2 -100%) when needed for the analyses. Optimal separation, recovery, and concentration of components were obtained by eluting with 1 ml each of 10, 20, 40, and 100% acetonitrile. Similar elutions of the acid fraction Sep-Paks were carried out with various concentrations of acetonitrile that were 5×10^{-3} M tetrabutylammonium phosphate.

Sequential Partioning

Another method of obtaining component fractions consisted of eluting loaded Sep-Paks first with 4 ml of an azeotropic mixture of methanol and 1,2-dichloropropane, and then with 4 ml ether. The azeotropic extract was evaporated and then extracted with cloroform. This yielded group 1 (chloroform insoluble) and group 2 (chloroform soluble) fractions. The ether extract from the Sep-Paks was evaporated and extracted with distilled water. This yielded group 3 (ether and water soluble compounds). The water

insoluble fraction was then solubilized with 1 M HCl. This fraction was basified with 2 M NaOH and extracted with ether. The base soluble fraction was separated as group 4 from the ether layer. After neutralizing with acetic acid, group 5 (amphoteric compounds) were isolated with ether. The HCl insoluble fraction in ether was neutralized with 5% aqueous $NaHCO_3$. After addition of NaCl, the aqueous layer was separated. Stronger acids (group 6) were precipitated with HCl and extracted from ether. To the organic phase from above was added 0.5% NaOH. Phenolics and weakly acidic compounds (group 7) were present in the aqueous phase, while neutral compounds (group 8) remained in the organic phase.

Identification Procedures

The compounds in the neutral extract were analyzed by gas liquid and high pressure liquid chromatography. Gas liquid chromatography (GLC) was carried out using a Perkin-Elmer Model Sigma 2B system equipped with a flame ionization detector. The extract was analyzed on 2 different capillary columns using identical conditions except for temperature (see below). The conditions were the following:

Injector Temperature:		200°C
Detector Temperature:		200°C
Flow N_2	:	0.5 cc/minute
Split Injection	:	Ratio undetermined

The columns used were of different polarity which allowed more certainty in the identification of compounds by co-chromatography. One was a 25 m x 0.02 mm ID OV 101 and was used at 70 - 200°C at 5°C advancement per minute. The other was 15 m x 0.02 mm ID SE 54 and was used at 70 - 200°C at 4°C advancement per minute. Quantification was carried out using fenchone as

the internal standard, and was corrected for individual response factors. These columns will separate monoterpenes and sesquiterpenes, as well as the phenylpropanoids (such as esdragol, eugenol, methyl-eugenol, and some of the phenylpropanoids listed in Figure 2).

Analysis of the extract obtained from the freezing out procedure was carried out using high pressure liquid chromatography. The extracts were dissolved in 60% CH_3CN (acetonitrile). These were then run under the following conditions:

10 μ Bondapak C_{18}, 25 cm long column (Waters)

Time Program Gradient: HPLC water for 5 minutes

0-40% Ch_3CN for 40 minutes

Hold for 10 minutes

40-100% Ch_3CN for 10 minutes

Flow Rate : 1 ml/minute

These conditions produced maximum separation of the extracts on HPLC, and has been used by us in other studies for the separation of sesquiterpene lactones, flavonoids, coumarins, and other natural products. Detection was done at 254 nm and 214 nm in series.

RESULTS

We began first with the samples obtained from the freezing-out method because this fraction appeared to contain large amounts of terpenoids which, as noted in the literature review, have not been extensively investigated. Analysis of the extract (5.76 g; 0.4% by weight of the H_2O) by gas liquid chromatography revealed the presence of greater than 50 components. 7 major monoterpenes (1.41 g; 0.094% by weight of the H_2O) accounted for 75% of that fraction and 24% of the total dissolved organics recovered by the freeze out method. Co-chromatography with authentic standards on 2 columns of different polarity indicated that they were p-cymene, cineole, citronellol, geraniol, citronellyl acetate, geranyl acetate and bornyl acetate, all monocyclic and acyclic monoterpenes and their acetates (Table 1). Figure 3 is a typical chromatogram of the volatiles found in this extract. In addition, 3 other monoterpenes, thymol, citronellal, and terpinene 4-ol, were found along with a sesquiterpene α-humulene. These were identified on only one GLC system.

Table 2 shows the terpene hydrocarbons of the 3 samples that were collected on 3 August 1982. It is very interesting to note that even though these samples were collected at exactly the same time (3 large mouth brown bottles were emerged in the streamwater in quick succession), they show different qualitative and quantitative patterns. In total, extract A contained more than 50 compounds, but the extract was dominated by bornyl acetate which recorded 61% of the total extract. For extract B more than 30 compounds were present, but geranyl acetate and geraniol were the dominant components amounting to 31% and 27%, respectively. Extract C contained more than 30 compounds, but was dominated by citronellyl acetate,

Table 1. Components of the composite extract obtained through the frozen-out method. Water was collected on 3 August 1982 in 3 bottles and composited in the laboratory.

Compounds	Quantity (mg)	% of Water (by weight)
p-Cymene	144	0.009
1,8-Cineole	31	0.002
Citronellol	190	0.013
Geraniol	223	0.015
Bornyl Acetate	361	0.024
Citronellyl Acetate	175	0.012
Geranyl Acetate	284	0.019
Total	1.41g	0.094

Figure 3. Typical GLC chromatogram of the volatiles found in the frozen-out extract.

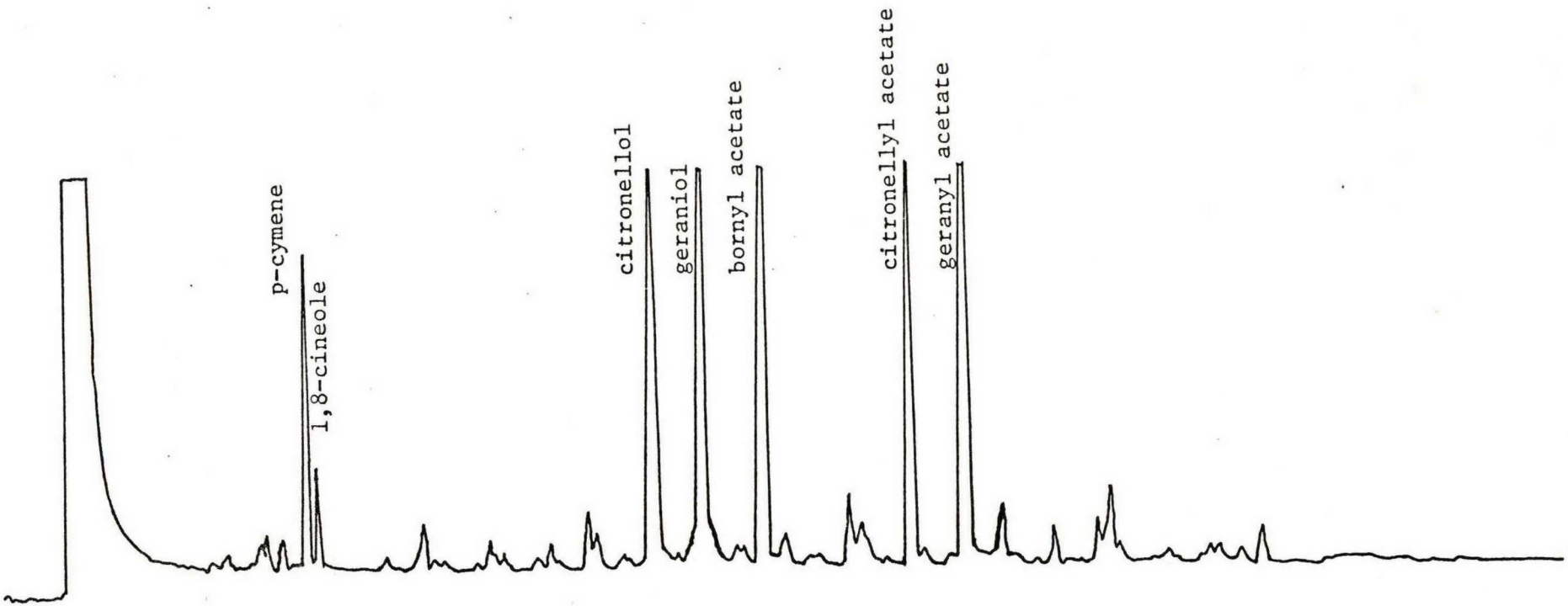


Table 2. Qualitative and quantitative variation in terpenes in streamwater samples collected 3 August 1983 in quick succession.

Terpene	Extract (% of Extract)		
	A	B	C
ρ-Cymene	2.5	-	16.0
Cineole	1.0	2.0	1.0
Citronellol	2.0	11.0	17.0
Citronellyl Acetate	-	6.9	20.0
Geraniol	0.5	27.0	0.5
Geranyl Acetate	-	31.0	2.0
Bornyl Acetate	61.0	-	-
Citronellal			t
Thymol	t		
Terpinolene 4-ol	t		
α-Humulene	t		

- = not present; t = trace amount present; blank = not known for certain if occurs in extract

citronellol, and p-cymene which amounted to 20%, 17%, and 16%, respectively.

Table 3 lists all of the compounds that were investigated. Of these 28, 11 were found in the frozen out extracts (see Tables 1 and 2).

High Pressure Liquid Chromatography of the Frozen Out Fraction

High pressure reverse phase chromatography was performed with the intent to determine the extent of the more polar components of the extract including phenolic material. The major peaks present co-chromatographed with authentic standards of the terpenes previously identified by GLC but the resolution was not as good. None of the component peaks coeluted with authentic standards of a limited amount of phenolics which we investigated. These included naringin, queretin, and carveol. This is not surprising since many of the flavonoids present as glycosides are likely to remain with the H_2O during our freezing out procedure. This extract however, may be expected to contain diterpenoid resin acid as well as condensed tannins. These classes of compounds may represent a significant portion of the 75% remaining unidentified organic in the extract but were not investigated here.

Liquid Chromatography of Other Fractions

Funds were expended before detailed analysis of the other fractions generated by the Sep-Pak and sequential elution methods could be carried out. However, in the preliminary analysis, elution of the acid Sep-Paks yielded 5 major components and 3 minor ones. The Sep-Paks containing the neutral compounds that remained in the organic phase (group 8) yielded 4 major components and 3 minor ones.

Table 3. Order of elution of authentic terpenoid standards under conditions described in the experimental procedures. All values are relative to -terpineol which was assigned a value of 1.0.

Terpenoid*	Retention Times on Columns	
	SE 54 (4°C/min)	OV 101 (5°C/min)
α-Pinene	0.388	
Camphene	0.413	
β-Pinene	0.465	
Myrcene	0.489	
Caproic Ethylate	0.508	
α-Phellandrene	0.515	0.530
Carene	0.534	0.542
α-Terpinene	0.545	
p-Cymene	0.545	0.542
1,8 Cineole	0.562	0.589
Limonene		
Cis Ocimene	0.587	
Terpinolene	0.723	
Camphor	0.861	
Citonellal		0.888
Terpinene 4-ol		0.960
α-Terpineol	1.000	1.000
Carveol	--	1.076
Citronellol	1.120	
Geraniol	1.200	1.107
Citronellyl Acetate	1.474	1.401
Geranyl Acetate	--	1.455
Longifolene	1.597	--
Methyleugenol	1.609	--
α-Humulene	1.712	--
p-Thymol	1.321	--
Boranyl Acetate	1.300	--
γ-Cadinene	1.809	--

* values expressed as relative to α-terpineol rention time

DISCUSSION

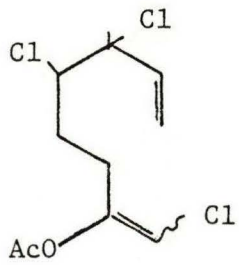
The major objective of determining some of the major components in the aspen-conifer streamwater was accomplished. Of the 28 compounds investigated, 11 were identified through co-chromatography using 2 columns differing in polarity on the gas chromatography system and a Bondapak C_{18} column on the liquid chromatography system. One of the important results, in addition, was the knowledge that volatiles are very common in the streamwater, particularly the terpenoids. This study has shown that there may be as many as 15 compounds in two other extracts that were run on the HPLC. All extracts are being refrigerated in the hope that additional funds can be obtained for the analysis of the other fractions.

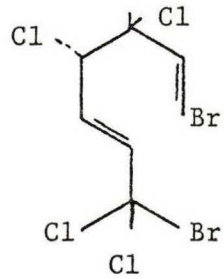
The efficiency of the various extraction or fractionating methods cannot be analyzed completely until the fractions generated from the other methods are analyzed in detail. However, it is very interesting to note the large difference that can be obtained in water samples that are collected at the same time (Table 3). Future studies involving monitoring dissolved organics in streamwater must deal carefully with the heterogeneity that is found in streamwater, and sampling regimes should be appropriately designed. This is perhaps, a major unexpected finding from this investigation. Preliminary analyses will be needed before a rigorous sampling design can be determined.

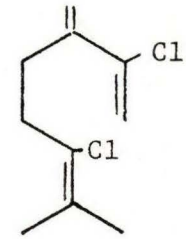
Under certain environmental conditions, various organic compounds will react differently with a particular chemical oxidant. Therefore, no general statement can be made concerning their oxidation by a particular chemical. The separation and identification of these constituents is therefore

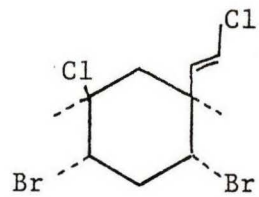
an essential step in the process of elucidating the pathway of their degradation in streamwater, or their reactivity in other processes. The routine use of chlorine in wastewater treatment plants produces substantial quantities of the oxidants Cl_2, HOCl, OCl, and HCl (Faust and Hunter, 1971). While it is not possible at this stage to predict the kinetics and the products of these oxidations with the terpenes identified in our study, it is interesting to note that halogenated terpenes (Figure 4) are found in marine environments where they are presumably synthesized by various algae. These are known to be toxic to fish and insects (Crews, 1977a,b; Faulkner et al., 1973). The possibility exists that under a water-treatment-plant chlorination environment, terpenes may give rise to a whole new class of chlorinated hydrocarbons, some of which may be toxic to humans. Further studies are needed to investigate in detail the possibility of this occurring.

Figure 4. Examples of halogenated terpenes found in Marine Algae.









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